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# Role of Flagella in Adherence, Internalization, and Translocation of *Campylobacter jejuni* in Nonpolarized and Polarized Epithelial Cell Cultures

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**Previous studies of *Campylobacter jejuni* have suggested that flagellin is an adhesin for epithelial cells and that motility is a virulence factor of this bacterium. The role of flagella in the interactions of *C. jejuni* with nonpolarized and polarized epithelial cells was examined with flagellar mutants. Flagellated, nonmotile (*flaA flaB*$^+$ Mot$^-$) and nonflagellated, nonmotile (*flaA flaB* Mot$^-$) mutants of *C. jejuni* were constructed by in vivo homologous recombination and gene replacement techniques. Both classes of mutants were found to adhere to cells of human epithelial origin (INT 407) equally well; however, on the basis of the percentage of the inoculum internalized, internalization of the *flaA flaB* Mot$^-$ mutants was decreased by factors ranging from approximately 30 to 40 compared with the parent. The *flaA flaB*$^+$ Mot$^-$ mutant was internalized by the INT 407 cells at levels six- to sevenfold higher than the *flaA flaB* Mot$^-$ mutants. Both classes of mutants, unlike the parent, were unable to translocate across polarized Caco-2 monolayers. These results indicate that flagella are not involved in *C. jejuni* adherence to epithelial cells but that they do play a role in internalization. Furthermore, the results suggest that either the motility of *C. jejuni* or the product of *flaA* is essential for the bacterium to cross polarized epithelial cell monolayers.**

The ability of a number of enteropathogenic bacteria to evade host defense mechanisms and cause disease by growth within or translocation across epithelial cell barriers is well documented (42). Through the use of cultured cells as model systems, *Escherichia coli*, *Salmonella* species, *Shigella flexneri*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* have been shown to enter, survive, and/or replicate within epithelial cell monolayers (3, 34). Translocation across polarized epithelial cell barriers by *Salmonella choleraesuis* and *Salmonella typhimurium* has also been demonstrated (11). Studies of *Campylobacter jejuni*, a significant cause of human enteritis, have indicated that penetration of the intestinal mucosa may also play a role in the pathogenesis of *C. jejuni*-mediated enteritis (8, 25).

Several studies have shown that *C. jejuni* adheres to cultured cells of human intestinal epithelial origin (INT 407) (19, 20, 26, 27, 31) and becomes internalized within these cells (21, 40). *C. jejuni* has also been shown to traverse monolayers of polarized human colonic carcinoma (Caco-2) cells (9, 22). Polarized Caco-2 cells, with apical and basolateral surfaces separated by tight junctions, express several markers characteristic of normal small intestine cells and have normal microvilli and a well-defined brush border (11, 16). Accordingly, they are thought to be more representative of intestinal cell architecture and function. The translocation of *C. jejuni* across such an epithelial cell barrier may reflect a pathogenic mechanism by which this organism can gain access to submucosal tissue, leading to the tissue damage and inflammation that is often associated with *C. jejuni* enteritis.

Factors produced by *C. jejuni* that have been suggested to play a role in adherence to epithelial cells include outer membrane proteins, flagella, and lipopolysaccharide (6, 10, 27, 29). Flagellin has been proposed as an adhesin in the binding of *C. jejuni* to cultured cells (27). Studies with colonization models have suggested that flagella play a role in the ability of *C. jejuni* to colonize the intestinal tracts of rabbits, suckling mice, and hamsters (1, 4, 31, 37). Expression of flagellin by *Campylobacter* species undergoes antigenic (14) and phase (4) variation, although not all *Campylobacter* isolates undergo both types of variation. Studies with rabbits (4) and human volunteers (2) have shown that there is a selection for flagellated *C. jejuni* in the intestinal tract, suggesting a role in pathogenesis. The genes encoding flagellin have been isolated from strains of *C. jejuni* (32) and *Campylobacter coli* (13). *C. jejuni* 81116, a strain which undergoes phase, but not antigenic, variation, carries two flagellin genes in a tandem orientation (32). Expression of only one of the *fla* genes in *C. jejuni* 81116, *flaA*, is apparent under usual in vitro growth conditions (32). In contrast to *C. jejuni* 81116, *C. coli* VC167, which also carries two flagellin genes in a tandem orientation and undergoes antigenic but not phase variation, expresses both *flaA* and *flaB* regardless of the antigenic type of flagellin produced; however, *flaA* is expressed at higher levels than *flaB* in *C. coli* VC167 (13). The basis for variable flagellin gene expression is not understood.

In the presently described study, in vitro cell assays for adherence and penetration of cultured cells of human intestinal epithelial origin (INT 407) were used to determine the

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role of *C. jejuni* flagella in these processes. Two classes of *C. jejuni* flagellar mutants were constructed and examined for the ability to adhere to and enter cultured human epithelial cells (INT 407) and to cross a polarized cell monolayer of human colonic epithelial origin (Caco-2). The data indicate that flagellin itself is not a primary adhesin but that flagella and/or motility is required for the internalization of *C. jejuni*.

## MATERIALS AND METHODS

**Bacteria.** *C. jejuni* 81116 was isolated by Newell et al. (31) from a patient with diarrhea and has been passaged many times in the laboratory. *C. jejuni* 81116 was grown at 37°C on Mueller-Hinton (MH) agar plates (GIBCO-BRL, Gaithersburg, Md.) in BBL GasPak jars containing BBL CampyPak Plus packets (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.). Flagellin gene mutants of *C. jejuni* 81116 were grown under identical conditions, with the exception that kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo.) was added to the MH agar plates at 50 μg/ml. Motility agar was prepared as follows. MH medium was supplemented with 0.4% agar and autoclaved. One half of the medium was cooled to 42°C and a suspension of the bacteria was added. The plates were allowed to solidify at room temperature and incubated at 37°C as outlined above. The other half of the MH–0.4% agar medium was used to pour plates directly. Bacteria were then streaked onto these plates and incubated as described above. *E. coli* DH5α, used for the growth of the recombinant plasmids and as a control in the Caco-2 cell assays, was grown in LB medium (10 g of Bacto-tryptone, 5 g of yeast extract [GIBCO-BRL], 5 g of sodium chloride [Sigma] per liter). The medium was supplemented with ampicillin (50 μg/ml; Sigma) and kanamycin (50 μg/ml) as appropriate.

**Manipulation and analysis of *C. jejuni* DNA.** Chromosomal DNA from *C. jejuni* 81116 was purified by the hexadecyltrimethyl ammonium bromide (Sigma) method as described by Wilson (43). Plasmid DNA was purified on an ethidium bromide-CsCl density gradient after preparation of a cleared lysate as described by Sambrook et al. (33). Two oligonucleotides, 5′-ATGGGATTTCGTATTAACACAAATGTTG CA-3′ and 5′-AATGTTGCAGCATTAAATGCTAAAGCAA AT-3′, were synthesized (Operon Technologies, Alameda, Calif.) on the basis of the DNA sequence of the flagellin genes of *C. coli* (13). The two oligonucleotides (which overlapped by 9 nucleotides) were 30 bases in length and were derived from the region from 1 to 51 of the *C. coli flaA* nucleotide sequence (13). The oligonucleotides were radiolabelled by the polynucleotide kinase (New England Biolabs, Beverly, Mass.) forward reaction with [$\gamma$-$^{32}$P]ATP (New England Nuclear [NEN], Boston, Mass.) as described by Sambrook et al. (33). Southern hybridization analysis of chromosomal DNA of *C. jejuni* 81116 and colony hybridization of *E. coli* recombinant colonies with the two oligonucleotides were done under conditions described by Dalbadie-McFarland et al. (5). Southern hybridization of *C. jejuni* chromosomal DNA and recombinant plasmid DNA with nick-translated DNA probes was performed under high-stringency conditions as described in the GeneScreen Plus protocol manual (NEN). DNA fragments were nick translated with [$\alpha$-$^{32}$P]dCTP (NEN) with a nick translation kit from Bethesda Research Laboratories (BRL) or Promega Corp. (Madison, Wis.). The *C. jejuni* 81116 DNA was digested with restriction endonucleases under conditions recommended by the suppliers (New England Biolabs and BRL). Restriction fragments containing the *C. jejuni flaA* gene fragment were isolated from agarose gels by a glassmilk-sodium iodide procedure (Bio 101, La Jolla, Calif.). The isolated DNA was ligated into plasmid pUC18 (38, 44) which had been digested with *Pst*I (BRL) and treated with calf intestinal alkaline phosphatase (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) and with T4 DNA ligase (BRL) in an overnight incubation at 14°C under conditions described by the supplier. The DNA was then transformed into *E. coli* DH5α by electroporation with a Bio-Rad Gene Pulser with pulse controller (Bio-Rad Laboratories, Richmond, Calif.) as described by Dower et al. (7).

**Adherence and internalization assays.** Stock cultures of INT 407 (human embryonic intestine; ATCC CCL 6) cells were obtained from the American Type Culture Collection (Rockville, Md.). INT 407 cells were cultured in Eagle's minimal essential medium (EMEM; Whittaker Bioproducts, Inc., Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS). The cultures were incubated in a humidified, 5% $CO_2$ incubator at 37°C. The bacteria used in the experimental assays were harvested from MH agar plates with phosphate-buffered saline (PBS), centrifuged at 6,000 × *g* for 10 min, and suspended in EMEM supplemented with 1% FBS. Semiconfluent monolayers of cultured INT 407 cells were rinsed once with prewarmed EMEM supplemented with 1% FBS. The media overlaying the monolayers were removed, and the cultures were inoculated with $5 \times 10^7$ CFU of *C. jejuni* in a volume of 0.5 ml, with a ratio of bacteria to epithelial cells of approximately 500:1. The plates were centrifuged at 600 × *g* for 10 min and then incubated for 0.5 h at 37°C in a 5% $CO_2$ incubator to allow adherence to the INT 407 cells. The monolayers were then rinsed three times with PBS, and the cells were lysed with 0.5% (wt/vol) sodium deoxycholate (Fluka Chemical Corp., Ronkonkoma, N.Y.). Adherent bacteria were quantified by plating serial dilutions of the lysates on MH agar plates and counting the resultant colonies.

To examine internalization, INT 407 cell monolayers were washed and inoculated with *C. jejuni* as described above. The plates were then incubated for 3 h at 37°C in a 5% $CO_2$ incubator to allow the bacteria to enter the INT 407 cells. The monolayers were then rinsed three times with EMEM and incubated in EMEM containing 1% FBS and 250 μg of gentamicin (Sigma) per ml for 3 h at 37°C in a 5% $CO_2$ incubator. After incubation, the infected monolayers were washed three times with PBS and lysed by the addition of 0.5% (wt/vol) sodium deoxycholate (Fluka). The number of internalized bacteria was determined as described for the adherence assay.

Statistical analyses of the adherence and internalization data were done with the program INSTAT (Graphpad, San Diego, Calif.). The ratios of the number of adherent and internalized bacteria to the number of bacteria inoculated in each well were calculated and multiplied by 100 to obtain percentages. The percentage values were logarithmically (base 10) transformed to eliminate the effects of positive skewing and the resultant heterogeneity of variances. The log-transformed values were analyzed by one-way analysis of variance. Evaluations of significant differences among pairwise comparisons were performed with *t* tests that were adjusted with the Bonferroni correction for multiple comparisons (35) and a significance level corresponding to $P \leq 0.05$.

**Bacterial translocation across polarized epithelial cell monolayers.** Caco-2 cells (human colonic carcinoma; ATCC HTB37) were obtained from the American Type Culture Collection and were cultured in EMEM supplemented with 10% FBS in a humidified, 5% $CO_2$ incubator at 37°C.

Transwell filter units (Costar, Cambridge, Mass.) containing 0.33-$cm^2$ porous membranes (pore size, 0.3 μm) were seeded with $10^5$ Caco-2 cells and placed in a well of a 24-well tissue culture tray containing 1 ml of EMEM supplemented with 10% FBS. The medium in contact with the apical and basolateral cell surfaces was changed every 72 h. The polarized Caco-2 monolayers were used between days 10 and 14 of incubation. The integrity of the monolayer and tight cell junctions was assessed by measurement of the electrical resistance across the monolayer with a Millicell-ERS apparatus according to the manufacturer's instructions (Millipore Corp., Bedford, Mass.).

Bacteria were harvested from agar plates and suspended in EMEM containing 1% FBS. The bacterial suspensions ($10^7$ CFU) were added to the apical side of the Caco-2 monolayer cultures following the removal of the overlaying medium. The filter units were then placed in the wells of a 24-well tissue culture tray containing 1 ml of fresh medium per well. The cultures were incubated in a humidified, 5% $CO_2$ incubator at 37°C. At 1-h intervals, the filter units were transferred to a 24-well tissue culture tray containing fresh, prewarmed medium. Viable bacteria in the basolateral (lower) medium were quantified by plating dilutions and counting the resulting colonies on agar plates.

To determine the association of the bacteria with the polarized monolayer cells, metabolically labelled *C. jejuni* was used. Bacteria were harvested from agar plates, washed with PBS, and suspended in EMEM without methionine. [$^{35}$S]methionine (1,200 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added at a concentration of 25 μCi/ml, and the bacteria were incubated with agitation for 1 h at 37°C in a 10% $CO_2$ incubator, followed by the addition of a 1/10 volume of 100 mM unlabelled L-methionine. The bacteria were washed three times with EMEM by centrifugation and suspended in EMEM supplemented with 1% FBS. Equal numbers of labelled bacteria were then added to the apical medium of the Caco-2 cells as described above. Following a 4-h incubation, the Transwell filters were washed three times with EMEM and the amount of radioactivity associated with the filters was determined by liquid scintillation spectrometry.

**Immunoblotting.** Whole-cell extracts of *C. jejuni* 81116 and the flagellar mutants were separated on 12.5% polyacrylamide-sodium dodecyl sulfate minigels by the discontinuous buffer system described by Laemmli (24). Proteins were electroblotted to polyvinylidene fluoride membrane filters (Immobilon P; Millipore) with the buffers described by Towbin et al. (36). The filters were incubated for 18 h at 4°C in PBS–Tween 20–20% FBS, with either anti-flagellin monoclonal antibody M4AB4 or a rabbit polyclonal anti-flagellin serum which had been raised against a purified flagellin preparation from *C. jejuni* 81116. Bound antibodies were detected with rabbit anti-mouse or goat anti-rabbit peroxidase-conjugated immunoglobulin G and 4-chloro-1-naphthol as the chromogenic substrate.

## RESULTS

**Cloning of flagellin genes and construction of flagellar mutants.** Two oligonucleotide probes derived from the 5′-terminal DNA sequence of flagellin A (*flaA*) gene of *C. coli* (13) were found to hybridize to a 5.0-kb *Pst*I fragment from *C. jejuni* 81116 chromosomal DNA. DNA corresponding to a 4.0- to 6.0-kb region of a *Pst*I digest of *C. jejuni* 81116 DNA was purified by agarose gel electrophoresis and used to clone the 5.0-kb *Pst*I fragment into pUC18. A recombinant plasmid carrying this 5.0-kb *Pst*I fragment was identified by hybridization to the oligonucleotide probes. Restriction enzyme mapping, Southern hybridization analyses with the two oligonucleotides, and limited DNA sequence analyses revealed that the *fla* gene resided near the 3′ end of the cloned fragment and that the *fla* open reading frame proceeded towards the 3′ end of the fragment (Fig. 1B). Comparison to the published DNA sequence of *C. jejuni* 81116 flagellin genes (Fig. 1A) (32) indicated that the 5.0-kb *Pst*I fragment contained approximately 1.4 kb of the *flaA* coding region and approximately 3.7 kb of DNA 5′ to the *flaA* gene but not the 3′ end of the *flaA* gene itself. A 1.27-kb *Bgl*II-*Pst*I fragment (Fig. 1C) containing *flaA* sequences but lacking the 5′ start of *flaA* and the 3′ end of *flaA* was subcloned into pUC18. This plasmid was subsequently used for *fla* mutant construction. The *Campylobacter* plasmid pILL550 (23) was digested with *Eco*RI and *Hin*dIII, and the 1.4-kb kanamycin resistance ($Km^r$) gene marker was isolated by agarose gel purification. The $Km^r$ DNA fragment was inserted into the cloned *flaA* gene sequences at a unique *Eco*RV site, such that there was approximately 850 bp and approximately 400 bp of *flaA* sequence flanking the 5′ and 3′ ends of the $Km^r$ gene, respectively (Fig. 1C). The resulting plasmid was transferred to *C. jejuni* 81116 by electroporation and acted as a suicide plasmid to deliver the interrupted *flaA* gene into the *C. jejuni* chromosome through allelic exchange of the flagellin gene sequences of the chromosome and the plasmid. Potential insertional mutants were identified by the acquisition of kanamycin resistance.

**Analysis of flagellar mutants.** Kanamycin-resistant *C. jejuni* 81116 colonies (~100) were screened for the presence of flagella by the staining method of Heimbrook et al. (15). Isolates which appeared not to possess flagella by this method were chosen for analysis by transmission electron microscopy (data not shown). Two isolates which did not have flagella as judged by transmission electron microscopy were chosen for further study and were designated GRK5 and GRK7. A third isolate, termed GRK17, was kanamycin resistant but possessed flagella as judged by transmission electron microscopy. However, the flagella of GRK17 appeared to be slightly shorter than the flagella produced by the parent 81116 (data not shown) and appeared similar to the flagella of the *flaA flaB*$^+$ *C. jejuni* strain described by Wassenaar et al. (41). The motility of the GRK strains was examined by phase-contrast microscopy and by motility (soft) agar assays, and all three GRK strains, including GRK17, were nonmotile. Whole-cell lysates of *C. jejuni* 81116, GRK5, GRK7, and GRK17 were then analyzed by immunoblotting with a monoclonal antibody to *C. jejuni* flagellin. As shown in Fig. 2A, while the monoclonal antibody recognized flagellin ($M_r$, 62,000) in the lysate from the parent 81116, strains GRK5, GRK7, and GRK17 did not produce flagellin detectable by this antibody. A parallel blot was probed with a polyclonal antiserum which had been raised against a purified flagellin preparation of *C. jejuni* 81116 (Fig. 2B). No flagellin was detectable by this antiserum in extracts of GRK5 and GRK7; however, immunoreactive flagellin ($M_r$, 62,000) was present in the GRK17 extract.

Chromosomal DNA preparations from *C. jejuni* 81116, GRK5, GRK7, and GRK17 were analyzed by Southern hybridization to examine the structure of the altered *fla* gene regions. As noted above (Fig. 1A) (32), *C. jejuni* 81116 has two, similar, tandemly oriented copies of the flagellin gene. Chromosomal DNA was digested with restriction enzymes known not to cut within the $Km^r$ gene fragment. The DNA

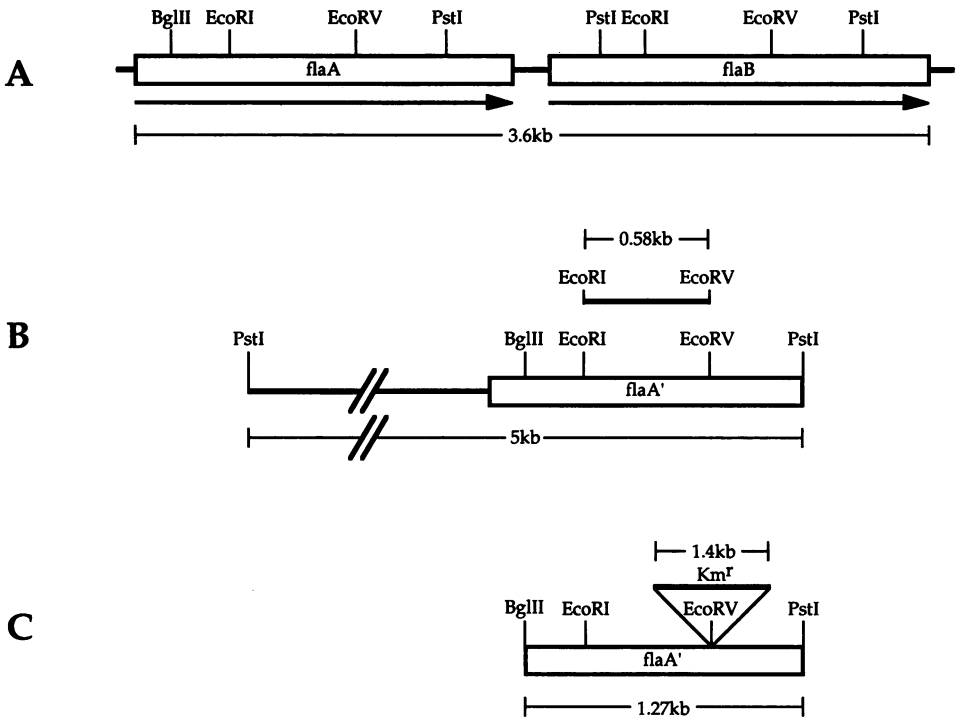


FIG. 1. Schematic representation of flagellin genes of *C. jejuni*, the cloned *flaA* fragment, and the recombinant fragment used in the construction of *fla* mutants. (A) Organization of flagellin genes in *C. jejuni* 81116 as described by Nuijten et al. (32). (B) A 5.0-kb *Pst*I clone containing a 1.4-kb fragment of *flaA* gene isolated from *C. jejuni* 81116. A 0.58-kb *Eco*RI-*Eco*RV fragment of *flaA* gene used as a probe in the Southern hybridization analysis shown in Fig. 3 is overlined. (C) A 1.27-kb fragment of *flaA* used for *fla* mutant construction. Insertion of a 1.4-kb Km$^r$ gene from pIll550 (23) at the *Eco*RV site of *flaA* also shown. A 1.4-kb Km$^r$ gene was used as a probe in the Southern hybridization analysis shown in Fig. 4. Restriction enzyme sites shown in Fig. 1 were confirmed by Southern hybridization with both *flaA* and *flaB* DNA probes. The genes and fragments shown are not drawn to scale.

probe for these analyses was a 0.58-kb *Eco*RI-*Eco*RV *flaA* fragment which contained only *flaA* sequences (Fig. 1B). Figure 3A (lane 1) shows the hybridization pattern expected for the parent 81116 DNA digested with *Pst*I: a 5.0-kb fragment which corresponds to the *flaA* gene plus the region 5′ to the *flaA* gene region, and a 1.2-kb fragment of *flaB*. In

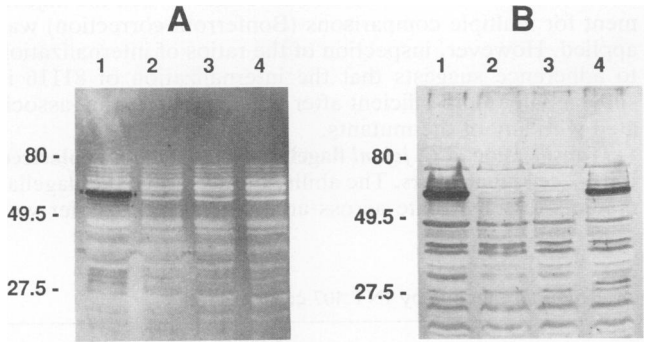


FIG. 2. Detection of flagellin production by *C. jejuni* 81116 and *fla* mutants by immunoblotting. Whole-cell extracts of the parent and *fla* mutants were electrophoresed in 12.5% gels, electroblotted, and reacted with anti-flagellin monoclonal antibody M4AB4 (A) and with anti-flagellin polyclonal serum (B) as described in Materials and Methods. Lanes 1, *C. jejuni* 81116; lanes 2, *C. jejuni* 81116-GRK5; lanes 3, *C. jejuni* 81116-GRK7; lanes 4, *C. jejuni* 81116-GRK17. The positions of the molecular mass standards are indicated and are given in kilodaltons.

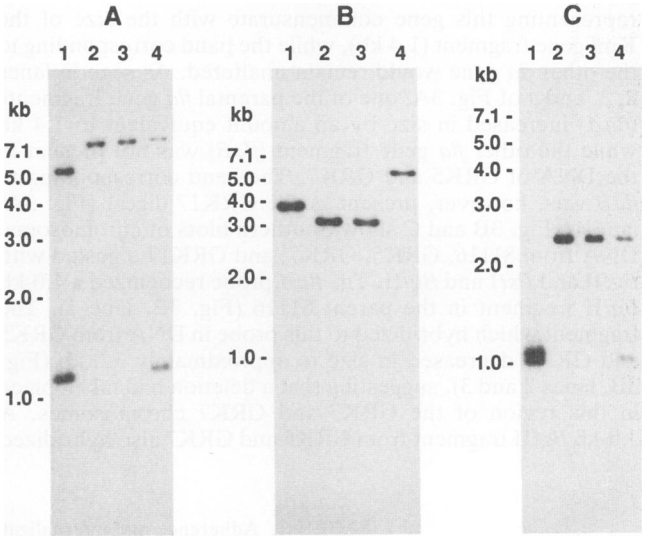


FIG. 3. Southern hybridization analysis of the flagellin gene structure of *C. jejuni* 81116 and *fla* mutants. Chromosomal DNA was digested with *Pst*I (A), *Bgl*II (B), and *Pst*I and *Bgl*II (C), electrophoresed in a 0.7% agarose gel, and blotted to a GeneScreen Plus membrane (NEN). Hybridization with a radiolabelled 0.58-kb *Eco*RI-*Eco*RV fragment of the *flaA* gene of *C. jejuni* 81116 (Fig. 1B) was performed as described in Materials and Methods. Lanes 1, *C. jejuni* 81116; lanes 2, *C. jejuni* 81116-GRK5; lanes 3, *C. jejuni* 81116-GRK7; lanes 4, *C. jejuni* 81116-GRK17. The positions of the size standards are indicated and are given in kilobases.

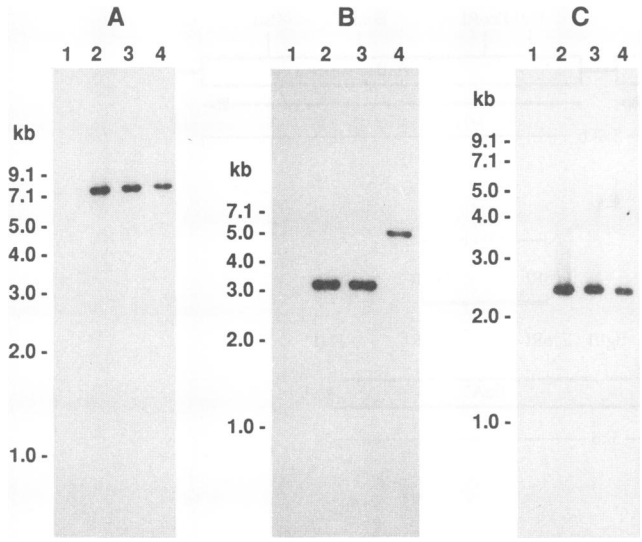


FIG. 4. Southern hybridization analysis of the flagellin gene structure of *C. jejuni* 81116 and *fla* mutants. Chromosomal DNA was digested with *Pst*I (A), *Bgl*II (B), and *Pst*I and *Bgl*II (C), electrophoresed in a 0.7% agarose gel, and blotted to a GeneScreen Plus membrane (NEN). Hybridization with a radiolabelled 1.4-kb *Eco*RI-*Hin*dIII fragment of plasmid pILL550 (23) which encodes a kanamycin resistance gene was performed as described in Materials and Methods. Lanes 1, *C. jejuni* 81116; lanes 2, *C. jejuni* 81116-GRK5; lanes 3, *C. jejuni* 81116-GRK7; lanes 4, *C. jejuni* 81116-GRK17. The positions of the size standards are indicated and are given in kilobases.

the case of the insertional mutants, if the Km$^r$ gene fragment had integrated into either of the two *fla* gene copies, the Southern blot would show an increase in size of the band representing this gene commensurate with the size of the Km$^r$ gene fragment (1.4 kb), while the band corresponding to the other *fla* gene would remain unaltered. As seen in lanes 2, 3, and 4 of Fig. 3A, one of the parental *fla* gene fragments (*flaA*) increased in size by an amount equivalent to 1.4 kb while the other *fla* gene fragment (*flaB*) was not present in the DNA of GRK5 and GRK7. The band corresponding to *flaB* was, however, present in the GRK17 digest (Fig. 3A, lane 4). Fig. 3B and C show Southern blots of chromosomal DNA from 81116, GRK5, GRK7, and GRK17 digested with *Bgl*II and *Pst*I and *Bgl*II. The *flaA* probe recognized a 4.0-kb *Bgl*II fragment in the parent 81116 (Fig. 3B, lane 1). The fragment which hybridized to this probe in DNA from GRK5 and GRK7 decreased in size to approximately 3.0 kb (Fig. 3B, lanes 2 and 3), suggesting that a deletion had taken place in this region of the GRK5 and GRK7 chromosomes. A 3.0-kb *Bgl*II fragment from GRK5 and GRK7 also hybridized with the Km$^r$ gene probe (Fig. 4B, lanes 2 and 3), indicating that this fragment, which hybridized with the *flaA* gene probe, contained the Km$^r$ gene insertion. The *Bgl*II fragment which hybridized to both the *flaA* gene probe and the kanamycin probe in GRK17 was increased in size compared with the parent 81116 (Fig. 3B, lane 4; Fig. 4B, lane 4), indicating that an insertion had taken place in the *flaA* gene in the GRK17 chromosome. As seen in Fig. 3C, the *Pst*I-*Bgl*II digests revealed that the band corresponding to *flaA* in the parent *C. jejuni* 81116 (1.1 kb [lane 1]) had increased in size in all three GRK isolates, while the *flaB* band (1.0 kb [lane 1]) was present only in GRK17 (Fig. 3C, lanes 2, 3, and 4). The Southern blot with the Km$^r$ gene probe results confirmed that the Km$^r$ gene fragment was integrated into the *flaA* gene of GRK5 and GRK7 (Fig. 3 and 4A and C) and GRK17 (Fig. 3 and 4A, B, and C). Southern hybridization analysis was also performed to verify that no plasmid sequences other than the *fla* and Km$^r$ gene fragments were integrated into the *C. jejuni* 81116 chromosome (data not shown). Collectively, the Southern hybridization analyses indicated that both the flagellin genes were disrupted in GRK5 and GRK7 (*flaA flaB*) and that only *flaA* was disrupted in GRK17 (*flaA flaB*$^+$). This latter observation suggests that the flagella of GRK17 are composed solely of flagellin derived from *flaB*.

**Interactions of *C. jejuni* flagellar mutants with epithelial cells.** To determine whether flagella or the flagellins play a role in the ability of *C. jejuni* to adhere to and/or enter human epithelial cells, *C. jejuni* 81116 and the isogenic *fla* mutants were assayed for their ability to bind and enter INT 407 cells. To enhance the association of the bacteria with the monolayers and to minimize motility-dependent effects, centrifugation was used to promote contact with the epithelial cells. No statistically significant differences in adherence to INT 407 cells between 81116 and the mutants were noted (Table 1). As shown in Table 1, 0.016% of the initial inoculum of *C. jejuni* 81116 was internalized into the INT 407 cells. A noninvasive control, *E. coli* DH5α, routinely shows internalization levels of 0.001 to 0.005% of the initial inoculum (data not shown). The internalization of the parent 81116 was significantly greater ($P < 0.05$) than that of the mutants GRK5 and GRK7 (Table 1). The analyses also revealed no statistically significant difference between the internalization exhibited by 81116 and the mutant GRK17 when the adjustment for multiple comparisons (Bonferroni correction) was applied. However, inspection of the ratios of internalization to adherence suggests that the internalization of 81116 is substantially more efficient after binding than is that associated with any of the mutants.

**Translocation of *C. jejuni* flagellar mutants across polarized Caco-2 cell monolayers.** The ability of the parent and flagellar mutants to translocate across an epithelial cell barrier was

TABLE 1. Adherence and internalization of *C. jejuni fla* mutants by INT 407 cells

| Strain | Inoculum | Adherence | Internalization | % of inoculum internalized | I/A[a] |
|---|---|---|---|---|---|
| Parent (81116) | $1.4 \times 10^8$ | $2.0 \times 10^4 \pm 0.3 \times 10^4$ | $2.3 \times 10^4 \pm 1.5 \times 10^4$ | $0.016 \pm 0.011$ | 1.15 |
| GRK5 | $1.0 \times 10^8$ | $4.7 \times 10^4 \pm 0.6 \times 10^4$ | $5.0 \times 10^2 \pm 0.1 \times 10^2$ | $0.0005 \pm 0.00001$[b] | 0.01 |
| GRK7 | $2.0 \times 10^7$ | $2.0 \times 10^4 \pm 0.8 \times 10^4$ | $8.0 \times 10^1 \pm 3.0 \times 10^1$ | $0.0004 \pm 0.00015$[b] | 0.004 |
| GRK17 | $5.0 \times 10^7$ | $1.3 \times 10^4 \pm 0.6 \times 10^4$ | $1.5 \times 10^3 \pm 0.2 \times 10^3$ | $0.003 \pm 0.0004$ | 0.115 |

[a] Ratio of number of bacteria internalized to number of adherent bacteria.
[b] Internalization of the mutants GRK5 and GRK17 was significantly different ($P < 0.05$) from that of the parent 81116 as judged by two-tail *t* tests with the Bonferroni correction.

examined with polarized Caco-2 cell monolayers. Translocation across the cell monolayer was assayed by determining the number of bacteria in the basolateral medium after apical inoculation. *E. coli* DH5α was used as a negative control, as this bacterium does not penetrate the Caco-2 monolayers unless the tight junctions have been disrupted or the monolayers have been damaged. Infection of the polarized Caco-2 monolayer with the parent 81116 and the GRK mutants did not result in the loss of transepithelial resistance of the monolayer during the course of the assay. *C. jejuni* 81116, GRK5, GRK7, and GRK17 (approximately $10^7$ CFU) were added to the apical surface of the polarized Caco-2 cells. The number of parental *C. jejuni* 81116 present in the basolateral medium increased at each hour, from $1.2 \times 10^3$ at 1 h to $1.2 \times 10^5$ at 5 h postinoculation. At no time during the assay did the *flaA flaB* mutant strains GRK5 and GRK7 or the *flaA flaB*$^+$ mutant GRK17 cross the Caco-2 cell barrier. To determine whether GRK5, GRK7, and GRK17 were unable to traverse the Caco-2 cells because they were unable to associate with the cells, *C. jejuni* 81116 and the three GRK mutant strains were metabolically labelled with [$^{35}$S]methionine and added to the apical surface of the polarized Caco-2 cells and the amount of radioactivity associated with the monolayers was determined after a 4-h incubation. When compared with the parent 81116, the number of each of the GRK strains associated with the monolayers was reduced by a factor of 4 to 5 (81116, 9.28% of initial inoculum; GRK5, 2.28%; GRK7, 1.66%; and GRK17, 1.98%). The decrease in the number of the *fla* mutants associated with the Caco-2 cells is likely due to the lack of motility of these bacteria. However, there were sufficient numbers of these mutant strains in contact with the cells to expect ready detection of bacteria in the underlying medium if the GRK strains were able to translocate across the cell barrier after establishing contact.

## DISCUSSION

Previous work from one of our laboratories has demonstrated that *C. jejuni* is capable of entering human epithelial cells (INT 407) and traversing polarized epithelial cell monolayers of human colonic origin (Caco-2 [19, 22]). *C. jejuni fla* mutants were constructed and utilized to further and more precisely define the role of flagella in the interaction of *C. jejuni* with such epithelial cells in vitro. With respect to the genetic structure of the mutants, the generation of the deletion of a portion of the *fla* gene region in the insertional mutation protocol was not unexpected. The selection of insertion of the Km$^r$ gene into the *C. jejuni* chromosome required an in vivo recombination event at a site at which there are two regions of DNA over 1,700 bp in length with over 95% homology between them. During the integration of the Km$^r$ gene, one could envision intragene base pairing resulting in the excision of the *flaB* gene during the reformation of the double-stranded DNA. While such events accommodate the loss of *flaB* in the formation of GRK5 and GRK7, the events leading to the formation of the mutation in GRK17 are not understood at this time.

Using the mutants, we found that aflagellated and flagellated, nonmotile *C. jejuni* adheres to cultured human epithelial cells as readily as the wild-type parent but was deficient in the ability to enter the epithelial cells, indicating a role for flagella or flagellin in internalization. In addition, our findings suggest that either motility per se or structural attributes of the *flaA* gene product are necessary for *C. jejuni* translocation across polarized epithelial cell monolayers. The potential role of flagella in the pathogenesis of enteritis caused by *C. jejuni* has been previously examined with naturally occurring *fla* variants and *fla mutant C. jejuni* produced by recombinant DNA techniques (1, 4, 28, 31, 41). Studies by Caldwell et al. (4) and Aguero-Rosenfeld et al. (1) have indicated that there may be selection for the presence of flagella during experimental infection of rabbits (4) and hamsters (1). Black et al. also found that there was a change from *fla* to *fla*$^+$ in human volunteers initially infected with aflagellated *C. jejuni* (2). McSweegan and Walker reported that flagella of *C. jejuni* may act as adhesins for epithelial cells in vitro (27), and Newell et al. found that flagellated, nonmotile *C. jejuni* attached to epithelial cell monolayers more efficiently than the parent strain or nonflagellated variants (31). These latter authors suggested that an adhesin is associated with the flagella and that active flagella are directly involved in only a weak association with the host cell. However, Morooka et al. reported that motility, and not just the presence of flagella, is required for colonization by this organism in the suckling mouse gut model (28). The possibility that flagella or flagellin is an essential adhesin is rendered less likely, given our findings that nonflagellated *C. jejuni* adheres to INT 407 cells as well as wild-type, flagellated bacteria. Consistent with our observations are the findings of Moser and Hellman, who reported that only 10 to 33% of binding to isolated murine small intestine cells was dependent on the presence of flagella (29). Further, Fauchere et al. (10) have reported that *C. jejuni* outer membrane proteins that bind to HeLa cells reside in fractions containing 26,000- to 30,000-$M_r$ proteins and not in those fractions containing flagellin.

Wassenaar et al. have used techniques similar to those described in this report to create *fla* mutants for use in studies of the invasion of epithelial cells by *C. jejuni* (41). Their studies were restricted to the use of nonpolarized cell cultures and assays for internalization. They found that when the bacteria were not centrifuged onto the monolayer, the entry or internalization of *flaA* mutants of *C. jejuni* 81116 was reduced by a factor of 100 compared with that in the parent. However, when the bacteria were centrifuged onto the monolayer, the level of penetration by the *flaA* mutants increased by 30-fold. A *flaA*$^+$ *flaB* Mot$^+$ mutant exhibited levels of internalization almost equivalent to that of the parent and which were unchanged by centrifugation of the bacteria onto the monolayer. The authors concluded that flagella composed of the FlaA protein are required for invasion of INT 407 cells and that motility is a major factor involved in cell invasion. However, they did not examine the possibility that adherence was affected. Our studies can be interpreted as formally excluding the possibility that the mutations in flagellar genes affect internalization by preventing adherence. The finding that antibodies directed against flagella have no effect on the adherence of *C. jejuni* to INT 407 cells (18a) further suggests that any adhesive attributes of flagella or flagellin are not essential for adherence of *C. jejuni* to cultured epithelial cells.

Recently, Jones et al. have reported that the direction of rotation of the flagella of *S. typhimurium* plays a role in the ability of this bacterium to invade HEp-2 cells (17). The authors reported that a smoothly swimming mutant of *S. typhimurium* exhibited increased levels of internalization, while a mutant locked in a tumbling type of motion and a flagellated, Mot$^-$ mutant exhibited decreased internalization compared with that of the wild-type parent. Our finding that flagellated, nonmotile *C. jejuni* (*flaA flaB*$^+$ GRK17) is internalized by INT 407 cells at a level reduced by a factor of 6 to

7 compared with that of the parent strain when the ratio of internalized to adherent bacteria is examined might suggest that motility plays a role in this process. Alternatively, the *flaA* gene product per se may be required for *C. jejuni* to be internalized by the INT 407 cells. Discrimination of these two possibilities will require isolation of a *flaA*$^+$ Mot$^-$ *C. jejuni* mutant, which we have as yet been unable to isolate. It is noteworthy, however, that GRK17 is internalized by the INT 407 cells at levels 10-fold higher than the nonflagellated mutants and, based on the percentage of the inoculum internalized, cannot be statistically distinguished from the parent. This result suggests that the flagellar structure plays some role in the internalization process of *C. jejuni* that is independent of motility.

Jones et al. have described a three-stage model of invasion of HeLa cells by *S. typhimurium* (18). Initially, the bacteria are brought into contact with the HeLa cells through motility, resulting in a reversible binding of the bacteria to the cells. The reversible binding in turn facilitates the binding of the bacterium in an irreversible manner, probably through a specific bacterial adhesin. Only irreversibly bound bacteria are then internalized. Similarly, *C. jejuni* may require the presence of flagella and motility to initiate the internalization process. For example, nonflagellated *C. jejuni* may not allow the shift from reversible to irreversible binding even when brought into contact with cells by centrifugation. Flagellin, while not the adhesin responsible for the irreversible binding, may still mediate initial, transient binding to the surface of the cultured cell and allow another adhesin to establish the irreversible binding required for internalization. However, flagella alone are unlikely to be sufficient to promote internalization, since maximal internalization of *C. jejuni*, like *S. typhimurium*, also requires de novo protein synthesis (12, 19).

It has been previously shown that *C. jejuni* cells pass through and between confluent polarized Caco-2 cells (9, 22). Our findings indicate that either motility or the *flaA* gene product or both appear to be essential for translocation across the polarized monolayer, since GRK5 and GRK7 (*flaA flaB* Mot$^-$) and GRK17 (*flaA flaB*$^+$ Mot$^-$) mutants were unable to cross the cell barrier. Although other enteric pathogens are known to translocate across polarized epithelial cells in vitro (11) and polarized epithelial cells are thought to better approximate in vivo conditions than conventionally cultured epithelial cells, the relationship between this phenomenon and the pathogenesis of disease is unclear. Indeed, studies have suggested that M cells may be the preferred target of bacterial entry for *C. jejuni* (39) and *S. flexneri* (30, 40). Irrespective of the likely or preferred target cell during in vivo symptomatic infection, the observations presented herein support the proposal that both flagella and associated motility are likely important pathogenic determinants. Motility may be key to the bacteria's ability to penetrate the submucosa, and while not an adhesin, flagellin may be required for the internalization of this organism.

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